

A Model Procedure for the Transfer of TLC Pharmaceutical Product Screening Methods Designed for Use in Developing Countries to Quantitative HPTLC–Densitometry Methods

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Summary. Transfer of four rapid thin-layer chromatography (TLC) screening methods used to detect substandard and counterfeit pharmaceutical products to quantitative high-performance TLC (HPTLC)–densitometry methods is demonstrated. These methods for acetaminophen, acetylsalicylic acid, ibuprofen, and chlorpheniramine maleate are contained in a Compendium of methods developed by Kenyon and Layloff for use in countries with limited resources. The new quantitative methods use Merck HPTLC silica gel 60 F₂₅₄ glass plates, automated standard and sample application, and automated densitometry for detection, identification, and quantification. Standard and sample solution preparation and application procedures for obtaining calibration curves and bracketed samples are described. The HPTLC plates give better efficiency, selectivity, and resolution than TLC, and the new methods overcome the deficiencies in technology related to manual application and visual zone comparison that do not allow the Compendium TLC procedures to support regulatory compliance actions. These transferred methods can be fully validated according to International Conference on Harmonization (ICH) guidelines or by interlaboratory studies if their applications require. The approach described can be used to transfer the remaining Compendium methods as well as the GPHF [Global (formerly German) Pharma Health Fund E.V.] Minilab kit TLC screening methods.

Key Words: high performance thin layer chromatography, HPTLC, densitometry, pharmaceutical product screening, drug analysis, counterfeit drugs, transfer of TLC methods to HPTLC–densitometry

Introduction

Thin-layer chromatography (TLC) based systems for pharmaceutical product screening analysis have been reviewed [1, 2]. One of these systems is the portable Minilab kit [3] that was developed by the Global (formerly German) Pharma Health Fund E.V. (GPHF) primarily for use in developing countries to help detect mislabeled, substandard, and counterfeit products. The kit contains the reagents, materials, and equipment necessary to

perform analyses of more than 40 pharmaceutical products with detailed manuals describing its use.

A similar semiquantitative TLC screening system for pharmaceutical product analysis by operators with limited training in field locations such as ports of entry, pharmacies, and distribution centers has been described in a Compendium by Kenyon and Layloff with detailed individual methods for 67 pharmaceutical products [4]. The methods allow the determination of whether the active pharmaceutical ingredient (API) is the one listed on the label and whether the content is the amount specified. For detection, the products are viewed as dark zones on a bright green fluorescent background under a battery-operated 254-nm UV lamp. Products without a UV chromophore are detected by dipping the plate in an iodine-KI or ninhydrin solution. The TLC analysis is based on use of one dosage unit to prepare the sample concentration; a tablet is ground and the entire contents transferred to the container used for dilution. Two reference concentrations are applied to the plate manually with a micropipette, that is, 85 and 115 or 120% representing the upper and lower specification limits for the dosage form, and the sample solution representing 100% is applied between the reference solutions. The detected zones, which should have the same R_F values to indicate qualitative identification, are examined visually to determine whether the size/intensity of the sample zone is between the size/intensity of the standard (reference) zones and, therefore, within the specification.

The purpose of this research was to develop a model approach for the transfer of the TLC screening methods to quantitative HPTLC-densitometry methods using the same group of solvents as in the Minilab and Kenyon TLC methods, which includes acetone, ammonium hydroxide, ethanol, ethyl acetate, glacial acetic acid, hydrochloric acid, methanol, sulfuric acid, and toluene. These solvents are readily available and relatively inexpensive, and they have a relatively low toxicity unlike chloroform or other halogenated solvents. In addition to using an HPTLC plate in place of a TLC plate, automated application of standard and sample solutions is used instead of manual application, and automated densitometry in the scanning mode is used for obtaining R_F values, recording densitograms, and quantification of the products in place of semiquantitative visual estimation. The skill level needed for using these transferred methods can be achieved after minimal additional training of operators performing the TLC methods [5], and the quantitative information obtained will better support regulatory compliance actions. This model approach is illustrated by the transfer of the TLC screening methods for analysis of three analgesic APIs, namely, acetylsalicylic acid, acetaminophen, and ibuprofen, and one antihistamine, namely, chlorpheniramine maleate.

General Experimental Procedures

Standard and sample solutions are prepared as described in the next section for the four active APIs using the following method: weigh the standard on an analytical balance accurate to ± 0.1 mg; grind the sample using a heavy-walled octagonal design mortar and pestle (Fisher Scientific, Pittsburgh, PA, Part No. 12-950AA); transfer the standard and sample into a 50- or 100-mL volumetric flask using about 30 or 70 mL of solvent, respectively, and magnetically stir and sonicate for 10 min each to dissolve the analyte; dilute to the line with the solvent; filter a portion of the sample solution into a vial using a 5-mL plastic syringe (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an Acrodisc 13-mm syringe filter (Pall Life Sciences, Ann Arbor, MI, USA), or allow the undissolved inactive ingredients to settle by standing; and use as is for HPTLC or dilute the clear solutions using volumetric flasks or calibrated pipettes as stated to prepare the 100% standard and sample solutions to be applied to the HPTLC plate.

The following standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): acetylsalicylic acid, No. 23,963-1, 99+% purity; acetaminophen, No. A3035, reference standard, 100.8% purity; ibuprofen, No. I110, >98% purity; and chlorpheniramine maleate, No. C-3025, >99% purity, Lot No. 011K02691V, purchased January, 2011. The generic analgesic and antihistamine samples analyzed were purchased from a local drug store. The same dosage strengths as specified in the Compendium methods were not available, and one was a caplet instead of a tablet; the sample solution preparation is adjusted according to the label value of the analyzed sample.

HPTLC is carried out in general according to the standardized procedures recommended by Reich and Schibli [6]. Merck silica gel 60 F₂₅₄ HPTLC glass plates (20 \times 10 cm, Part No. 5642-6) are used as received without prewashing. The specified sample and standard aliquot volumes are applied to plates in the form of bands with a Linomat 4 spray-on applicator (CAMAG, Muttensz, Switzerland) equipped with a 100- μ L syringe using the following settings: band length 6 mm, application rate 4 s μ L⁻¹, table speed 10 mm s⁻¹, distance between bands 4 mm, distance from the left edge of the plate 18 mm, and distance from the bottom of the plate 1.5 cm.

After the applied zones have dried, the plates are developed to a distance of 7 cm beyond the bottom of the plate in a CAMAG HPTLC twin-trough chamber containing 10 mL of the specified mobile phase in each trough and a saturation pad (Analtech, Newark, DE, USA) in the rear trough; the chamber is pre-equilibrated with the vapors of the mobile phase for 15 min before insertion of the plate into the front trough. The ambient

temperature in the laboratory was approximately 20°C and the relative humidity was approximately 12% during method development.

Developed plates are dried in a fume hood under a hair dryer for 5 min, and the areas of the product zones are measured immediately by absorption remission (reflectance) densitometry using a CAMAG Scanner 3 with the UV light source set at 254 nm, slit dimensions 4.00 × 0.45 mm Micro, and scanning rate 20 mm s⁻¹. The winCATS software automatically creates a calibration curve by linear regression correlating the weights of the standard zones to their scan areas and interpolates the weight of a bracketed sample zone from the curve based on its area. The result of the sample assay is calculated using the following equation: % = (experimental weight/theoretical weight predicted by the label declaration) × 100.

Results

The four quantitative HPTLC–densitometry methods that were developed by the transfer approach are as follows:

Ibuprofen Tablet, 200 mg

Sample solution: Grind 1 tablet and transfer the powder completely into a 50-mL volumetric flask with 30 mL of methanol–glacial acetic acid (49:1, *v/v*). Magnetically stir for 10 min, sonicate for 10 min, and dilute to the line with the same solvent mixture. Filter a portion of the solution into a vial. Dilute 1.50 mL of this solution with 8.50 mL of the solvent mixture to prepare the sample solution with a concentration of 6 µg/10 µL.

Standard solution: Dissolve 200 mg of standard in the solvent mixture in a 50-mL volumetric flask with shaking to complete dissolving. Mix 1.50 mL of this solution with 8.50 mL of the solvent mixture to prepare the 100% standard solution (6 µg/10 µL).

Sample and standard application: Apply 7, 9, 11, and 13 µL of the 100% standard solution (4.20–7.80 µg) and 10 µL of the sample solution in duplicate (6 µg theoretical based on the label value).

Development: Mobile phase, toluene–ethyl acetate–acetic acid (17:13:1, *v/v/v*); development time 13 min, $R_F = 0.62$.

Densitometry: Scan at 254 nm; linear calibration equation, $y = 746.7 + 551.1x$, where y = scan area and x = analyte (ibuprofen) weight, $r = 0.996$.

Assay results: The applied samples gave 5.78 and 5.64 μg when their scan areas were interpolated from the calibration curve, representing 96.3 and 94.0% relative to the label value, respectively.

Acetylsalicylic Acid Tablet, 325 mg

Sample solution: Treat one tablet as described above for ibuprofen, except mix 1 mL of the filtered solution with 9 mL of methanol–glacial acetic acid (49:1, *v/v*) to prepare the sample solution with a concentration of 6.50 $\mu\text{g}/10\ \mu\text{L}$.

Standard solution: Dissolve 600 mg of standard in the same solvent mixture in a 50-mL volumetric flask with shaking. Mix 0.50 mL of this solution with 9.50 mL of the same solvent mixture to the 100% standard solution equal to 6 $\mu\text{g}/10\ \mu\text{L}$.

Sample and standard application: Apply 7, 9, 11, and 13 μL of the 100% standard solution (4.20–7.80 μg) and 10 μL of the sample solution in duplicate (6.50 μg theoretical).

Development: Mobile phase, toluene–ethyl acetate–acetic acid (17:13:1, *v/v/v*); development time 15 min, $R_F = 0.38$.

Densitometry: Scan at 254 nm; linear calibration curve equation, $y = 2066.4 + 1359.1x$, $r = 0.999$.

Assay results: The applied samples gave 6.05 and 6.11 mg when the scan areas were interpolated from the calibration curve, representing 93.1 and 94.0% relative to the label value, respectively.

Acetaminophen (Paracetamol) Caplet, 500 mg

Sample solution: Dissolve 1 ground caplet in 95% ethanol with stirring and sonication in a 100-mL volumetric flask. After filtration, transfer 1 mL of this solution into a 25-mL volumetric flask and dilute to the line with 95% ethanol. Mix 1 mL of this solution with 9 mL of 95% ethanol in a vial to prepare a final concentration of 0.0200 $\mu\text{g}/10\ \mu\text{L}$.

Standard solution: Weigh 500 mg of standard and follow the same procedure as for the sample except shaking alone is sufficient for complete dissolution in the first step. After the 1:25 and 1:10 dilutions, the 100% standard concentration is 0.0200 $\mu\text{g}/10\ \mu\text{L}$.

Sample and standard application: Apply 7, 9, 11, and 13 μL of the standard solution (0.140–0.260 μg) and 10 μL of the sample solution in quadruplicate (0.200 μg theoretical).

Development: Mobile phase, ethyl acetate-methanol-conc. ammonium hydroxide (24:3:1, *v/v/v*); development time 16 min, R_F 0.51.

Densitometry: Scan at 254 nm; linear curve, $y = 463.4 + 23.3x$, $r = 0.997$.

Assay results: The applied samples gave 0.0175, 0.0177, 0.177, and 0.178 μg when their scan areas were interpolated from the calibration curve, representing 87.5, 88.5, 88.5, and 89.0% relative to the label value, respectively.

Chlorpheniramine Maleate Tablet, 4 mg

Sample solution: Dissolve one ground tablet in 40 mL of anhydrous ethanol, measured by pipette, in a 50-mL volumetric flask with stirring and sonication (1 $\mu\text{g}/10 \mu\text{L}$). Filter before application onto the layer.

Standard solution: Dissolve 100 mg of standard in anhydrous ethanol in a 100-mL volumetric flask with shaking. Dilute this solution 1:10 with the same solvent to prepare a 1 $\mu\text{g}/10 \mu\text{L}$ 100% standard solution. The standards degrade with time; use a fresh standard to avoid high assay results.

Sample and standard application: Apply 7, 9, 11, and 13 μL of the 100% standard solution (0.700–1.30 μg) and 10 μL of the sample solution in duplicate (1 μg theoretical based on the label value).

Development: Mobile phase, methanol-deionized water-glacial acetic acid (70:20:1); development time 45 min, R_F 0.46.

Densitometry: Scan at 254 nm; linear calibration equation, $y = -683.8 + 3.06x$, $r = 0.992$.

Assay results: The applied samples gave 1.06 and 1.11 μg when the scan areas were interpolated from the calibration curve, representing 106 and 111% relative to the label value, respectively.

Discussion

The TLC screening to HPTLC–densitometry quantitative method transfer procedure described for three analgesics and one antihistamine is a model that should be applicable to all of the other methods in the Compendium and Minilab. An effort was made to keep the method transfer procedures as direct as possible in terms of the solvents used for standard and sample solution preparation, mobile phase composition, and weights of standard and sample solutions applied to the plate. The mobile phase should give a compact, uniform analyte zone resulting in a sharp, symmetrical scan peak, and the R_F value should be 0.2–0.8 for maximum resolution of sample

components [7]. Fig. 1 shows a densitogram of acetylsalicylic acid as an example.

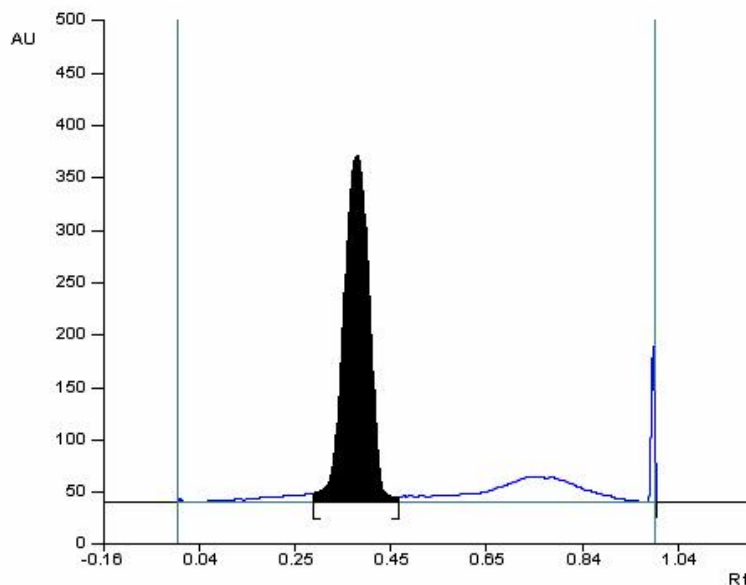


Fig. 1. Densitogram of 10 μL of acetylsalicylic acid sample solution representing 6.05 μg when its area was interpolated from the calibration curve

The application volumes of the prepared sample and standard solutions needed for successful visual comparison of the standard and sample zones are not given in the Compendium for any of the methods. When rapid screening methods were published by Kenyon et al. [8] for some of the tuberculosis pharmaceuticals covered in the Compendium, an application volume of 3 μL was given; therefore, a 3- μL application volume was assumed for the four Compendium methods that were transferred. This allowed calculation of the sample weight applied in 3 μL in TLC, and this weight in 10 μL was used as a guideline for the concentrations of the sample and 100% standard solutions to be prepared for HPTLC procedures. This guideline was suitable for predicting the calibration curves for ibuprofen and acetylsalicylic acid but not for acetaminophen and chlorpheniramine maleate. The 1 mg mL^{-1} concentrations suggested in the Compendium for these two products gave sample and standard zones on HPTLC plates that were too dark for establishing a good calibration curve and interpolating the sample weight when this guideline and our application regimen were followed. In order to obtain suitable results for these products, trial-and-error experimental dilutions based on published HPTLC-densitometry methods were required. Minilab TLC methods

specify a 2- μL application volume and the standard and sample solution concentrations, so a weight guideline can be calculated for first-try preparation of standard and sample solutions needed in the transfer of these methods to HPTLC.

The concentration of the 100% standard solution prepared for HPTLC is chosen so that a calibration curve covering the required content specification range of the products is achieved by applying 7, 9, 11, and 13 μL of the solution (70–130%). The number and range of the standards applied can be easily changed by adjusting the settings of the Linomat depending on the specific requirements of the developed methods. The Linomat applies initial zones in the form of bands rather than the round spots that are applied manually with a micropipette. Band-shaped zones are known to be ideal for densitometric quantification. The sample solution based on the label value was prepared so that the area of a zone resulting from application of 10 μL would be bracketed near the center of the calibration curve. An advantage of having a calibration curve is that the weight represented by 10 μL of the sample solution does not have to exactly match the weight of 10 μL of the standard solution, as long as it is bracketed by the calibration curve.

Application of different volumes of a single standard solution rather than the same volume from a series of different standard solutions is almost always used with the Linomat in creating a calibration curve. This volume-based calibration technique is recognized as valid for quantitative analysis by CAMAG [9] and the internationally recognized expert TLC/HPTLC pharmaceutical product analyst Elke Hahn-Deinstrop [10] if done properly. Application of different volumes saves much time in standard preparation and should not cause poor results if the single standard is properly prepared. However, multiple standards cannot always be applied from a single standard solution; for example, if it is desired to prepare a three-point calibration curve of 75, 100, and 115%, three standard solutions must be prepared and equal volumes applied because the Linomat cannot apply the needed fractional 7.5 and 11.5 μL volume aliquots along with a 10- μL aliquot from a 100% standard solution.

The Merck silica gel 60 F₂₅₄ HPTLC glass plates used have a 150–200 μm layer thickness of silica gel with 5–7 μm particle size and 0.74–0.84 mL g^{-1} pore volume. The Merck silica gel TLC plastic sheets used in the early 1990s when the Compendium methods were developed had silica gel manufactured by the same process and the same layer thickness as current HPTLC plates, but the particle size was higher (10–12 μm) in the TLC plastic sheets [11]. The composition of the binder in the two layers is proprietary information not available from Merck. The general similarity of the TLC

plastic sheets and HPTLC glass plates should lead to direct transfer of the mobile phase for most of the Compendium methods, and we found this to be true for all but the chlorpheniramine maleate method; for this product, the Compendium mobile phase (methanol-acetic acid (22:1)) gave an R_F value below 0.2 (0.080), and a more polar mobile phase (methanol-water-glacial acetic acid (70:20:1); R_F 0.46) was designed based on the principles of mobile phase optimization [12] without the use of any solvents not in the inventory of the Compendium methods. Merck also offers silica gel 60 F HPTLC aluminum sheets (Part No. 5548) with the same layer as the HPTLC glass plates, and the same results should be obtained with both products.

Scanning was carried out at 254 nm using the deuterium lamp of the scanner. At this wavelength, the fluorescent indicator (phosphor) impregnated in the layer emits visible green fluorescence, and compounds that have a UV absorption maximum at or near 254 nm, including the four products analyzed, partially absorb the irradiating light and are detected as dark zones on the fluorescent background. Greater amounts of a given product diminish (quench) the layer fluorescence to a greater degree, leading to darker zones and larger scan areas and the ability to construct a calibration curve. Acetaminophen absorbs 254 nm UV light especially strongly, and as a result its calibration curve covers the lowest weight range (0.140–0.260 μg) of the four studied products. For a product without a UV chromophore, iodine-KI or ninhydrin reagent can be applied for detection using a CAMAG dip tank or a Kontes (Vineland, NJ USA) Chromaflex sprayer. Iodine is a universal reagent with relatively low sensitivity for organic compounds; the detected zones are not stable, and a validated densitometric determination is not possible. Ninhydrin selectively reacts with compounds containing an amino (NH_2) group. Densitometric determinations can be carried out successfully using ninhydrin detection, as has been shown for the quantification of the amino acid arginine in dietary supplement tablets [13].

The WinCATS software provides linear and second-order regression, but linear regression was used in all of the transferred methods above. Slightly different sample weights are obtained depending upon the type of regression; for example, for the ibuprofen method described above, the polynomial regression calibration equation is $y = -780.2 + 1087.2x - 44.7x^2$, $r = 0.999$; interpolated weights are 5.64 and 5.50 μg ; and the respective assay results are 94.0 and 91.2%. It is impossible to know whether these results are more or less accurate than the 96.3 and 94.0% results obtained with the linear regression (above) unless an accuracy validation study is carried out by analyzing a standard reference material with a known quantity of analyte, comparing results with a second well-characterized method, or

performing a standard addition analysis. Fig. 2 shows the linear calibration curve for acetylsalicylic acid as an example.

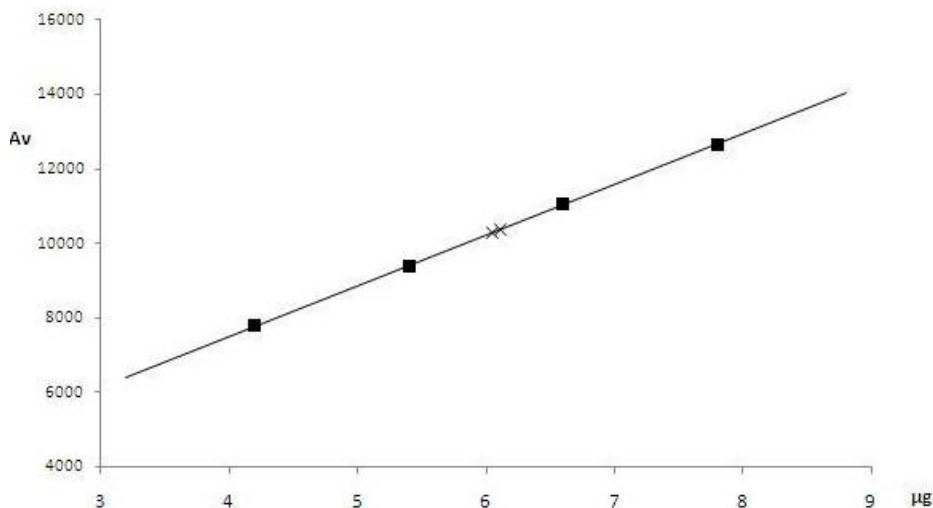


Fig. 2. Linear regression calibration curve for acetylsalicylic acid in the range of 4.20 µg (70%) to 7.80 µg (130%). The squares represent the four standard zones and the crosses the two sample zones

Nineteen tracks are available on each 20 × 10 cm plate for application of samples and standards. We applied four standard aliquots and duplicate aliquots of the sample on five adjacent tracks in three of the methods developed above; the duplicate samples provide an indication of the precision of the results. In the acetaminophen analysis, four aliquots of one sample were applied with four standards on a plate, and the relative standard deviation of the sample areas was found to be 0.61%. This confirms that the layer is uniform and the procedures of initial zone application and densitometric scanning are highly precise. Plates can be easily cut to other sizes, such as 10 × 10 cm, using a glass cutter to conserve plates in situations where all available tracks are not needed for an analysis.

All assays gave percentages ranging from 87.5 to 111% relative to the label value, indicating that all were within the specification limits of the four APIs. These results did not prove the accuracy of the methods; the accuracy determination, if needed, would require a formal accuracy validation study.

No additional peaks were detected in any sample densitogram, indicating that none of the inert ingredients (excipients) present in the four formulations analyzed quenched the fluorescence of the layer, and that

there were no impurities or degradation products present above the limit of detection (LOD). The inactive ingredients in the formulations were povidone, starch, and stearic acid for acetaminophen; dicalcium phosphate dihydrate, glyceryl triacetate, hypromellose, starch, and talc for acetylsalicylic acid; colloidal silicon dioxide, corn starch, croscarmellose sodium, hypromellose, iron oxides, microcrystalline cellulose, stearic acid, and titanium dioxide for ibuprofen; and corn starch, D&C yellow No. 10, lactose, and magnesium stearate for chlorpheniramine maleate.

These procedures were developed to demonstrate how to transfer from TLC screening to HPTLC quantitative methods. The HPTLC–densitometry results are not compromised by the limitation in technology related to the ability of operators to manually apply the sample and standard initial zones or visually compare zone sizes/intensities [14]. Interlaboratory studies [5] can now be carried out on these unofficial screening methods that have been converted to quantitative procedures to demonstrate their robustness and applicability for use in countries that have been using the Compendium methods. It has been shown that, with some additional training, some operators in these countries can competently perform HPTLC–densitometry [5, 14] and obtain much more information and better results compared to visual semiquantitative TLC methods. If necessary, full validation of the developed HPTLC–densitometry quantitative methods can be performed according to International Conference on Harmonization (ICH) guidelines for HPTLC pharmaceutical analysis [15, 16], including accuracy, precision (repeatability and intermediate precision), specificity, linearity, range, and robustness.

The advantages of HPTLC versus TLC procedures include narrow, symmetrical zones; greater sensitivity of zone detection; and higher accuracy and precision of densitometric analysis. The lower particle size and greater uniformity of HPTLC layers give better selectivity (α) and efficiency (N), leading to higher resolution approaching conventional column HPLC [14]. Advantages of HPTLC compared to column high-performance liquid chromatography (HPLC) are simplicity, ability to run multiple samples on one plate (high throughput), and better sustainability of the chromatographic system anywhere in the world [14].

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